

### **Remarks**

The Office Action mailed 3 July 2001 has been received and reviewed. The application is to be amended as previously set forth. All amendments and cancellations have been made without prejudice or disclaimer. Claims 1 through 11 are pending. New claims 12 through 16 have been added. Claim 4 has been cancelled without prejudice or disclaimer. All claims stand rejected. Reconsideration is respectfully requested.

### **Claim Rejections**

Applicants have amended the application to include the phrase "What is claimed is:" prior to recitation of the claims. (See, Appendix B and Appendix C).

### **Specification**

The specification was objected to for containing British spellings of certain words. Applicants have reviewed the specification and, pursuant to 37 C.F.R. §§ 1.121 and 1.125 (as amended to date) please enter the substitute specification in clean form and including paragraph numbers [0001] through [0071] attached hereto as Appendix B. A marked-up substitute specification to clearly identify amendments to the specification as required by 37 C.F.R. § 1.121(b)(3)(iii) is attached hereto as Appendix C. Reconsideration and withdrawal of the objection is requested.

### **Drawings**

Applicants submit, under separate cover, a letter to the Chief Draftsman concerning corrected drawings, including a better quality reproduction of the figures. Further, applicants respectfully submit that 37 C.F.R. § 1.84(b) no longer requires a petition to use photographs or special mounting.

### **35 U.S.C. §112**

Claim 6 was rejected under 35 U.S.C. §112, first paragraph, as assertedly containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to make and/or use the invention.

Specifically, the Examiner believed that applicants did not refer to a deposit of *Salmonella typhimurium* STM 2001 in the specification and therefore there was no assurance that all required deposits have been made under 37 CFR §1.801-1.809. Applicants respectfully submit that the specification refers to the deposit of *Salmonella typhimurium* STM 2001 in the specification on page 17, lines 9-11. Applicants have amended the specification to refer to the current address of the *Centraalbureau voor Schimmelcultures*. Further, applicants submit herewith an unsigned Budapest Treaty declaration as requested. (Office Action, page 4). A signed copy of the declaration will be forwarded upon receipt. Withdrawal of the rejection is thus requested.

Claims 2, 3, 5 and 7 were also rejected under 35 U.S.C. §112, first paragraph, as assertedly containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to make and/or use the invention. Specifically, it was asserted that the specification assertedly failed to provide guidance regarding how the *Salmonella* strain is mutated. Applicants respectfully submit that claims 2, 3, 5, and 7 are sufficiently enabled by the specification.

The essence of the invention as claimed is the use of mutated *Salmonellae* in (marker) vaccines. The specification describes several ways to construct these stable mutated bacteria, for example, by chemical or genetic mutation and the described methods are known, standard techniques. Similarly, the selection of non-motile bacteria from a pool of generated mutants was also performed by methods well known in the art.

Applicants respectfully submit that the specification, as filed, provides sufficient disclosure for generation of mutated bacterium through genetic mutations. As noted by the Examiner, a great deal is known of the flagellar biogenesis pathways (Office Action, page 7, line 23 through page 8, line 8) and, as noted in the Specification, these pathways are described in several publications (Specification, page 5, lines 5-9). Also, flagellin genes have been described for a great number of bacteria, including several *Salmonella* species (Specification, page 6, lines 4-15). Additionally, the flagellar antigenic determinants have been mapped (Specification, page 4, lines 20-21), and specific ways of mutating any of these genes or determinants are disclosed. (Specification, page 7, lines 25 through page 9, line 5). Using this prior art, any person skilled in the art could, by using standard techniques (See, e.g., Manlatis et al.: Molecular Cloning, a laboratory manual) and relying on the

wealth of information, create *Salmonella* with genetic mutations in the flagellar biogenesis pathway, resulting in the generation of flagella negative and/or non-motile bacteria. Subsequent methods of selecting non-motile *Salmonella* are well known in the art, for example, as disclosed, via light microscopy (Specification, page 16, lines 13-16), or through antibody selection (Specification, page 15, lines 22 to end, and page 16, lines 1-2).

Further, applicants respectfully submit that the specification, as filed, provides sufficient disclosure for generation of mutated bacterium through chemical mutations. Techniques for the generation of mutated bacteria through chemical mutagenesis rely on the generation of random mutations followed by selection of the desired mutants. Such chemical mutations are disclosed through a number of examples, and as set forth herein, the subsequent selection of non-motile bacteria employs standard techniques, some of which are cited in the application (supra).

In particular, Example 1 describes the creation of a mutant bacterium STM 2000 by methods of chemical mutagenesis (Specification, page 15, lines 6-20), followed by antibody selection (Specification, page 15, lines 22 to end and page 16, lines 1-12). Next, these bacteria were functionally characterized as being non-motile by light microscopy (Specification, page 16, lines 13-16). Using similar methods, and through a different chemical mutation technique, bacterial mutant STM 2001 was generated as is described, for example, in the Specification on pages 16, last line through page 17 end.

Claim 2 recites a mutation in a gene of the flagellar biogenesis pathway. As stated, applicants respectfully submit that the specification discusses genes of the flagellar biogenesis pathway and possible mutations therein. (*See, for example, Specification*, page 5, lines 5-25). Further, different mutation methods are also discussed in the specification at, for example, page 7, lines 5-14 and page 7, line 25 through page 9, line 5. Applicants respectfully submit that the specification provides sufficient guidance regarding how to make a mutation in a gene of the flagellar biogenesis pathway.

Claim 3 recites a mutation in a flagellin gene. As stated, applicants respectfully submit that the specification discusses flagellin genes and possible mutations therein. (*See, for example, Specification*, page 6, lines 4-26). Further, different mutations methods are also discussed in the specification, for example, page 7, lines 5-14 and page 7, line 25 through page 9, line 5. Applicants

respectfully submit that the specification provides sufficient support regarding how to make a mutation in a flagellin gene.

Claim 5 recites a heterologous gene. As stated, applicants respectfully submit that the specification discusses flagellin genes and possible mutations therein. (*See, for example, Specification*, page 6, lines 4-26). Further, insertion of a heterologous gene in a bacteria is also discussed in the specification, for example, page 11, lines 1-25. Applicants respectfully submit that the specification provides sufficient support regarding how to make a bacterium that carries a heterologous gene.

Claim 7 includes limitations of a mutated bacterium that lacks at least one antigenic determinant of flagellin or flagella. As stated above, the specification discusses genes of the flagellar biogenesis pathway and possible mutations therein. (*See, for example, Specification*, page 5, lines 5-25). Further, different mutation methods are also discussed in the specification, for example, page 7, lines 5-14 and page 7, line 25 through page 9, line 5. Applicants respectfully submit that the specification provides sufficient support regarding how to make a mutation in a gene of the flagellar biogenesis pathway. Further, the specification discusses flagellin genes and possible mutations therein. (*See, for example, Specification*, page 6, lines 4-26). Further, different mutations methods are also discussed in the specification, for example, page 7, lines 5-14 and page 7, line 25 through page 9, line 5. Applicants respectfully submit that the specification does provide sufficient support regarding how to make a mutation in a flagellin gene.

In conclusion, techniques for generating, characterizing, and selecting mutated bacteria are all well known and available in the art. The general techniques, as well as specific examples have been disclosed in detail in the application as filed. Accordingly, applicants submit that the generation of the claimed bacteria is sufficiently disclosed, enabling any person skilled in the art to obtain similar mutants. Additionally, applicants note that neither the generation or selection of the mutants, nor the methods employed to do so, are part of the presently claimed invention. The present invention is centered around the surprising finding that flagella-less *Salmonella* can be used as an effective vaccine. Reconsideration and withdrawal of the rejection of claims 2, 3, 5 and 7 is requested.

Applicants have cancelled claim 4 without prejudice or disclaimer. Some of the elements of claim 4 have been incorporated into claim 1 and applicants have amended claim 1 as proposed by the Examiner to spell out the genus and species at the first appearance in the claims.

Claim 6 was rejected for including the limitation “immunological characteristics”. Applicants respectfully submit that the term “immunological characteristics” is known and understood by those of skill in the art. Further, applicants notes that similar terms that are also known in the art are used in the specification including “immunogenic protein” (Specification, page 2, line 22) and “immunogenicity” (Specification, page 3, line 1 and page 3, line 20). As the objected terms is of similar character, applicants respectfully submit that no grounds exist for the objection to the limitation “immunological characteristics”. Reconsideration and withdrawal of the rejection is requested.

The Examiner also thought that the limitation “immunogenically effective amount” in claim 7 was unclear. Applicants respectfully submit that “immunogenically effective amount” is a term known and understood in the art. Further, applicants respectfully submit that the Specification discloses a “vaccine dose”. (Specification, page 12, lines 9-11). Additionally, examples 2, 3, and 4 describe a vaccine dose in terms of quantities that invoke an immunologic response, specifically by inducing a certain serum antibody-titer, and by conveying protection against disease upon challenge. Reconsideration and withdrawal of the rejection is requested.

### 35 U.S.C. §102

Claims 1 and 4-6 were rejected under 35 U.S.C. §102(b) as anticipated by Anderson (GB Patent 1,109,179). Applicants have canceled claim 4 and therefore the rejection is moot with respect to claim 4.

Applicants have amended claim 1 to recite a mutated bacterium selected from the group consisting of *Salmonella species typhimurium, enteritidis, choleraesuis, dublin, abortus-ovi, abortus-equie, derby, hadar, heidelberg, agona, and arizonaee* that in its wild type form carries flagella, said mutated bacterium lacking at least one antigenic determinant of flagellin or flagella found in its wild type form. Applicants respectfully submit that Anderson lacks any disclosure of a mutated bacterium selected from the group consisting of *Salmonella species typhimurium,*

enteritidis, choleraesuis, dublin, abortus-ovi, abortus-equii, derby, hadar, heidelberg, agona, and arizonae. Instead, Anderson only refers to non-motile strains of *Salmonella typhi* and *Salmonella paratyphi*. Further, Anderson lacks any disclosure of a bacterium that carries a heterologous gene as recited in claim 5. Accordingly, applicants respectfully submit that claims 1 and 5 avoid Anderson.

With respect to claim 6, applicants respectfully submit that the specification defines the bacterium strain that has been deposited under the terms of the Budapest Treaty with the *Centraalbureau voor Schimmelcultures* under accession-number CBS 108995. The specification states that this bacterium strain is a non-motile mutant of *Salmonella typhimurium*. (See, for example, Specification, page 16, line 26 through page 17, line 11). Accordingly, applicants submit that the claimed strain is not the same as the *Salmonella* strains disclosed in Anderson.

New claim 12 recites a vaccine for the protection of a subject against Salmonellosis comprising an immunologically effective amount of a mutated bacterium selected from the group consisting of the *Salmonella* species *typhi* and *paratyphi A* and *B* that in its wildtype form carries flagella, said mutated bacterium lacking at least one antigenic determinant of flagellin or flagella found in its wildtype form, or an antigenic material thereof and a pharmaceutically acceptable carrier. Applicants respectfully submit that independent claim 12 avoids the Anderson reference.

Anderson discloses a anti-typhoid or anti-paratyphoid vaccine including a non-motile strain of *Salmonella typhi* or *Salmonella paratyphi* which is devoid of flagella capable of producing antibodies to the H antigen. The 1968 reference discloses flagella-less killed *Salmonella typhi* and *S. paratyphi A* and *B* vaccines for human parenteral use. Applicants respectfully submits that such vaccines were proven ineffective soon after publication of the Anderson reference. Applicants note the publication of an article in 1975 which was co-authored by Anderson. See, Wahdan, M. et al., 1975: Controlled field trial of a typhoid vaccine prepared with a nonmotile mutant of *Salmonella typhi* Ty2; Bull. World Health Organ., vol. 52, p. 69-73. A copy of the Wahdan reference is provided herewith.

Wahdan clearly states, “[t]hese results suggest that TMN1 [Typhoid non motile 1] vaccine does not provide protection against typhoid fever, and that H antigen may be an important component of an effective vaccine. (Wahdan, summary (emphasis added)). Further, Wahdan

states, “[t]he evidence currently available strongly suggests that **there is a correlation between protection and high H antibody levels**. The results of this study support this view.” (Wahdan, page 72, right col., lines 14-17 (emphasis added)). As the Salmonella vaccine described by Anderson is not effective, applicants respectfully submit that the Anderson vaccine should be deemed non-existing. Therefore, applicants respectfully submit that Anderson cannot be considered a prior art reference with respect to the present application.

Anderson and Wahdan’s conclusions are consistent with the Specification (Specification, page 3, line 14 through page 4, line 7) which identifies four reasons why flagella were never considered to be effective marker antigens. The publications cited (all from more recent dates) further stress the then current belief that flagella are essential for an efficient immune response.

The common assumption that flagella could not be considered effective marker antigens is the reason that for 25 years, the only allowed human vaccine against typhoid fever was based on whole cell killed *S. typhi* bacteria of strain Ty21. More recently, an attenuated live oral Ty21a vaccine, and very recently, a subunit vaccine based on capsular polysaccharide O-antigens were licensed. The Ty21 and the Ty21a Salmonella strains both retain the flagella H-antigen, and have been proven to be very effective. See e.g., B. Ivanoff et al., 1994: Vaccination against typhoid fever: present status; Bull. World Health Organ. vol. 72, p. 957-971. A copy of the Ivanoff references and is provided herewith.

Applicants further submit that these statements regarding the H antigen are also supported by He et al. (cited by the Examiner) which teaches that the flagellar H-antigen is an important antigen that should not be removed. Indeed, He et al. use this flagellum as a base for presenting heterologous antigens to the immune system because it is such an effective immuno-stimulant. For example, He et al. states that the *Salmonella* flagella carry the H antigen, *one of the major antigens that elicit immune responses in the infected host*. (He., page 2406, left col., lines 11-13; page 2413, left col., lines 20-22)(emphasis added). Further, He et al. notes that “the mutated flagelin genes . . . can be used as convenient expression vectors of foreign epitopes with potential for vaccine development.” (He et al. page 2413, left col., lines 43-44).

In conclusion, applicants submit that *Salmonella* flagella are generally considered to be very important for generating an efficient immune response. The only publication describing otherwise

(Anderson) is over 30 years old, and refers to a vaccine that in practice was found to be non-functional by its own author. Consequently, applicants respectfully submit that Anderson actually teaches away from the present invention. Accordingly, applicants respectfully submit claims 1 and 5 and 6 and new claim 12 avoid Anderson.

Claims 1 through 6 were rejected under 35 U.S.C. §102(b) as anticipated by He et al. ("He"). He discloses mutating the *fliC* gene of *Salmonella muenchen* and expressing the mutated gene in flagella negative strains of *S. dublin*. He describes the insertion of (fragments of) the flagellin gene of *S. muenchen* into flagella-less mutants of *S. typhimurium* or *E. coli*, and *S. dublin*. The generation of stable mutants of flagella-less *S. typhimurium* or *S. dublin* is not disclosed. Also vaccines or even marker-vaccines of these flagella-less bacteria were employed to enable the detection of expression of the inserted *S. muenchen* flagella genes for subsequent study of flagellum stability in order to identify a suitable location for the insertion of a foreign epitope.

As described herein, He stresses the importance of the H-antigen for an effective immune response which is also why the flagellum is employed as an efficient inducer of an immune response to an inserted foreign epitope. Applicants therefore submit that He et al. essentially teaches the preservation of flagella as they are important immunogen, and does not teach the use of the mutated bacteria of claim 1 for use in vaccines. Claims 2 through 6 avoid He et al. for substantially the same reasons as claim 1.

With respect to claim 2, applicants respectfully submit that He lacks any disclosure wherein a bacterium lacks at least one antigenic determinant of flagellin or flagella due to a mutation in a gene of the flagellar biogenesis pathway. Instead, He only discloses mutations of a flagellin gene.

With respect to claims 3 and 5, applicants respectfully submit that He lacks disclosure of the limitations of claim 1 that are incorporated into claims 3 and 5.

With respect to claim 6, applicants respectfully submit that the specification defines the bacterium strain that has been deposited with the Centraalbureau voor Schimmelcultures under accession-number CBS 108995. The specification states that this bacterium strain is a non-motile mutant of *Salmonella typhimurium*. (See, for example, Specification, page 16, line 26 through page 17, line 11). Accordingly, applicants submit that the claimed strain is not the same as the *Salmonella* disclosed in He.

Claims 7 through 11 were rejected under 35 U.S.C. §102(b) as anticipated by Anderson (GB Patent 1,109,179). Anderson is discussed above and incorporated herein. Applicants respectfully submit that claims 7 through 11 avoid Anderson for substantially the same reasons as stated with respect to claim 1 herein.

Claims 7 through 11 are dependent from claim 1 and include the limitations of claim 1, as amended, of a mutated bacterium selected from the group consisting of *Salmonella species typhimurium, enteritidis, choleraesuis, dublin, abortus-ovi, abortus-equ, derby, hadar, heidelberg, agona, and arizonae* that in its wild type form carries flagella, said mutated bacterium lacking at least one antigenic determinant of flagellin or flagella found in its wild type form. Applicants respectfully submit that Anderson lacks any disclosure of a mutated bacterium selected from the group consisting of *Salmonella species typhimurium, enteritidis, choleraesuis, dublin, abortus-ovi, abortus-equ, derby, hadar, heidelberg, agona, and arizonae*. Instead, Anderson only refers to non-motile strains of *Salmonella typhi* and *Salmonella paratyphi*. Accordingly, applicants respectfully submit claims 7 through 11 avoid Anderson.

Further, applicants respectfully disagree with the Examiner's contention that the limitation "a vaccine for the protection of animals against Salmonellosis" does not provide patentable weight. Applicants respectfully submit that the preamble of a claim of a patent constitutes a limitation on the claim for the purpose of determining whether a claim is anticipated by a prior art reference. *Divisitech Corp. v. Century Steps, Inc.*, 850 F.2d 675, 7 U.S.P.Q.2d 1315 (Fed. Cir. 1988). Further, the limitations in the preamble are necessary to give meaning to the claim and properly define the invention. The teachings of the prior art should not be measured against all vaccines broadly, but vaccines suitable for use as claimed by the present invention. *In re Stencel*, 828 F.2d 751, 4 U.S.P.Q.2d 1071 (Fed. Cir. 1987). Applicants respectfully submit that the preamble of claim 7 imposes a functional limitation that must be considered. As Anderson further lacks any disclosure of a vaccine for the protection of animals against Salmonellosis, applicants respectfully submit that claim 7 avoids Anderson.

Claims 7 through 11 were rejected under 35 U.S.C. §102(b) as anticipated by He et al. He is discussed above and incorporated herein. Applicants respectfully submit that claims 7 through 11 avoid He for substantially the same reasons as claim 1.

Claims 7 through 11 are dependent from claim 1 and include the limitations of claim 1, as amended, of a mutated bacterium selected from the group consisting of *Salmonella species typhimurium, enteritidis, choleraesuis, dublin, abortus-ovi, abortus-equi, derby, hadar, heidelberg, agona, and arizona* that in its wild type form carries flagella, said mutated bacterium lacking at least one antigenic determinant of flagellin or flagella found in its wild type form.

He discloses mutating the *fliC* gene of *Salmonella muenchen* and expressing the mutated gene in flagella negative strains of *S. dublin*. He describes the insertion of (fragments of) the flagellin gene of *S. muenchen* into flagella-less mutants of *S. typhimurium* or *E. coli*, and *S. dublin*. The generation of stable mutants of flagella-less *S. typhimurium* or *S. dublin* is not disclosed and vaccines and marker-vaccines of these flagella-less bacteria were employed to enable the detection of expression of the inserted *S. muenchen* flagella genes for subsequent study of flagellum stability in order to identify a suitable location for the insertion of a foreign epitope.

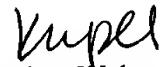
As described herein, He stresses the importance of the H-antigen for an effective immune response which is also why the flagellum is employed as an efficient inducer of an immune response to an inserted foreign epitope. Applicants therefore submit that He et al. essentially teaches the preservation of flagella as they are important immunogen, and does not teach the use of the mutated bacteria of claim 1 for use in vaccines. Accordingly, claims 7 through 11 avoid He.

Further, as previously stated herein, applicants respectfully disagree with the Examiner's contention that the limitation "a vaccine for the protection of animals against Salmonellosis" does not provide patentable weight. Applicants submit that a patent claim preamble constitutes a limitation on the claim for the purpose of determining whether a claim is anticipated by a prior art reference. *Divisitech Corp. v. Century Steps, Inc.*, supra. Further, the limitations in the preamble are necessary to give meaning to the claim and properly define the invention. The teachings of the prior art should not be measured against all vaccines broadly, but vaccines suitable for use as claimed by the present invention. *In re Stencel*, supra. Applicants respectfully submit that the preamble of claim 7 imposes a functional limitation that must be considered. As He further lacks any disclosure of a vaccine for the protection of animals against Salmonellosis, applicants respectfully submit that claim 7 avoids He.

### **Conclusion**

In view of the amendments and remarks, applicants respectfully submit that the amended claims define patentable subject matter. If questions should remain after consideration of the foregoing, the Examiner is kindly requested to contact applicant's attorney at the address or telephone number given herein.

Respectfully submitted,



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Date: January 3, 2002  
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**APPENDIX A**  
**VERSION WITH MARKINGS SHOWING CHANGES MADE**

1. (Twice amended) A mutated bacterium [of the genus *Salmonella*] selected from the group consisting of *Salmonella species typhimurium, enteritidis, choleraesuis, dublin, abortus-ovi, abortus-equi, derby, hadar, heidelberg, agona, and arizonaе* that in its wild type form carries flagella, said mutated bacterium lacking at least one antigenic determinant of flagellin or flagella found in its wild type form.
2. (Twice amended) The bacterium according to claim 1, wherein said bacterium lacks at least one antigenic determinant of [flagellan] flagellin or flagella, due to a mutation in a gene of the flagellar biogenesis pathway.

Please cancel claim 4 without prejudice or disclaimer.

6. (Amended twice) The bacterium according to claim 1, having the same immunological characteristics as the bacterium strain that has been deposited with the Centraalbureau voor Schimmelcultures under accession-number CBS 108995.

# **APPENDIX C**

**(MARKED UP VERSION)**

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**(Serial No. 09/749,025)**

PATENT  
Attorney Docket 5048US

NOTICE OF EXPRESS MAILING

Express Mail Mailing Label Number: \_\_\_\_\_

Date of Deposit with USPS: \_\_\_\_\_

Person making Deposit: \_\_\_\_\_

APPLICATION FOR LETTERS PATENT

for

**SALMONELLA VACCINE**

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TITLE OF THE INVENTION  
SALMONELLA VACCINE

CROSS REFERENCE TO RELATED APPLICATION

[0001] This application claims priority to, and is a continuation of, European Patent Application No. 99204564.1 filed on December 28, 1999, the contents of which are incorporated herein by this reference.

TECHNICAL FIELD

[0002] The present invention relates to *Salmonella* bacteria for use in a vaccine, to vaccines based upon these bacteria, to the use of such bacteria for the manufacture of a vaccine and to methods for the preparation of such vaccines.

BACKGROUND

[0003] Bacteria of the genus *Salmonella* are notorious for their pathogenicity in both man and animals. In the USA alone, on a yearly basis the number of humans suffering from *Salmonella* infections exceeds the two million cases. In most cases, the infection is caused by contaminated food. Well-known sources of infection are eggs (from both ducks and chickens), products containing eggs and [not sufficiently]insufficiently heated poultry and pig meat. Especially in infants, young children, elderly people and immune compromised patients, the ability to cope with such infections is low. Especially in these groups, the yearly death rate due to *Salmonella* infections is high. During the last few decades [the ]more efficient large-scale animal husbandry has led to an enormous increase in animal density. As a result, an increase [is]has been seen [of]in the number of animal infections and subsequent human infections caused by infected food. It is clear that animals are the main source of *Salmonella* infection. This source is very difficult to control. First[ of all], *Salmonella* infections in most cases cause no serious illness in healthy full-grown animals; these animals can carry the bacterium for a prolonged period. During that time, they are shedding the bacterium in their dung. This makes it practically impossible to avoid infection in the more vulnerable young animals. Secondly, many *Salmonella* species

[colonise]colonize several different host animal species. Some of the *Salmonella* species cause primary infections in specific hosts, whereas other *Salmonella* species are not restrictive at all. As a primary infectans, *S. typhi* and *paratyphi* are frequently associated with infection in man. *S. typhi* causes [diarrhoea]diarrhea, and as a result, dehydration. This infection is very frequently found in tropical areas. *S. dublin* is connected with cattle, specifically young animals, where it causes lethal infections in over 50 % of the cases. *S. abortus-equi* causes abortion in horses. *S. abortus-ovi* causes abortion in sheep. *S. choleraesuis* is the cause of lethal [diarrhoea]diarrhea in young pigs. *S. typhimurium* and *S. enteritidis* cause salmonellosis in poultry, pigs, cattle and rodents. *S. arizona* also causes disease in turkeys. Also, non-food animals such as reptiles suffer from *Salmonella* infections, such as *S. arizona*.

[0004] There clearly is a need for efficient *Salmonella* vaccines. Vaccines are needed to protect humans from *Salmonella* infections transmitted from man to man. Also, vaccines are needed to protect humans from food-borne *Salmonella* infections. And finally, vaccines are needed to protect animals against *Salmonella* infections.

[0005] Currently, several vaccines against various *Salmonella* species are commercially available. These vaccines, although sometimes efficacious from a vaccine point of view, [however]share a serious disadvantage. They generally induce an antibody population that equals that of an infection with wild type bacteria because they possess the same antigenic load as the wild type bacterium. Therefore, an analysis of the antibodies in the serum of a *Salmonella*-positive animal does not reveal why the animal is positive. This can be due to vaccination, but it can equally [well] be caused by infection with a virulent field strain. Therefore, if an animal is *Salmonella*-positive, it is considered to be infected. Therefore, it would be desirable to have a so-called marker vaccine. A marker vaccine is a vaccine that can be discriminated from wild type infection, [e.g.]for example, on the basis of a characteristic antibody panel, different from the antibody panel induced by wild type infection. A different antibody panel is induced[ e.g.], for example, when an immunogenic protein present [on]in a wild type bacterium is not present in a mutant that is used for vaccination[: the]. The host will then not make antibodies against that protein after vaccination.

[0006] A marker antigen has at least the following characteristics:

- => it can be deleted without severely impairing the viability of the bacterium.
- => it induces antibodies when present.
- => it does not, when present,[ not] contribute to the immunogenicity of the bacterium
- => its absence preferably contributes to attenuation.

[0007] When searching for suitable marker antigens, all known *Salmonella* antigens are to be considered. A known antigen found with all wild type *Salmonella* species with the exception of some *S. pullorum* and *gallinarum* subspecies is the flagellum. Examples of *Salmonella* species carrying flagella when in their wild type form are *S. typhimurium*, *enteritidis*, *choleraesuis*, *dublin*, *typhi*, *abortus-ovi*, *abortus-equi*, *paratyphi A* and *B*, *derby*, *hadar*, *heidelberg*, *agona* and *arizona*. Flagella are long structures protruding from the cell surface, that play an important role in motility and invasion of certain host cells. Flagella consist of long polymers of the protein called flagellin. It is known that these flagella induce high levels of antibodies. It is known that the absence of flagella does not significantly impair the viability of the bacterium outside the host: flagella-less mutants of practically all *Salmonella* species are known and can be grown *in vitro*. Nevertheless, flagellar proteins of *Salmonella* have never been contemplated as suitable markers, since they do not, or only partially, fulfill three of the four marker-requirements:

- although not essential for survival outside the host, they do play a significant role in bacterial survival and persistence in host macrophages, involved in inducing immunity. (Methner, U. and Barrow, P.A., Berl. Münch. Tierärztl. Wschr. 110: 391-396 (1997), Weinstein et al., Infect. Immun. 46: 819-825 (1984))
- they strongly contribute to the immunogenicity of the bacterium, a reason why they are [even] used as carrier proteins for short heterologous amino acid sequences (Joys, T.M., SAAS Bulletin: Biochem. & Biotech. 4: 56-59 (1991), Newton, S.M.C. et al., Science 244: 70-72 (1989))
- their absence does not contribute to attenuation (Lockman, H.A. and Curtiss, R., Infect. & Immun. 58: 137-143 (1990), Methner, U. and Barrow, P.A., Berl. Münch. Tierärztl. Wschr. 110: 391-396 (1997))

-- flagellated *Salmonella* bacteria inhibit the binding of other flagellated *Salmonellae*. Flagella-less *Salmonella* bacteria however show a reduced inhibition potential towards other flagellated *Salmonellae*, and therefore even increase the overall level of *Salmonella* infection. (Methner, U. and Barrow, P.A., Berl. Münch. Tierärztl. Wschr. 110:391-396 (1997), Weinstein et al., Infect. Immun. 46: 819-825 (1984)).

[0008] For these combined reasons, the flagellum was never considered to be a suitable deletable marker antigen for *Salmonella* vaccines.

#### DISCLOSURE OF THE INVENTION

[0009] Surprisingly, it was found now that contrary to the general assumption, the absence of flagella did not affect the vaccinating potential of *Salmonella*. This is especially surprising in view of the fact that in wild type *Salmonella* infected animals, large amounts of antibodies against the flagella are found, once more proving their antigenicity.

[0010] [It is an object] One aspect of the present invention [to provide] provides, for the first time, bacteria of the genus *Salmonella* that in their wild type form carry flagella but are no longer capable [to induce]of inducing antibodies against at least one antigenic determinant of flagellin or flagella, for use in a vaccine for the protection of humans and animals against Salmonellosis.

[0011] Flagellins are proteins of about 500 amino acids that comprise several antigenic determinants, i.e. there are several regions on the flagellins against which antibodies are raised in the host animal. These antigenic determinants have been identified and [localised]localized by T.M. Joys. (Joys, T.M., SAAS Bulletin: Biochem. & Biotech. 4: 56-59 (1991)). For the purpose of the present invention, bacteria that are no longer capable [to induce]of inducing antibodies against at least one antigenic determinant of flagellin or flagella are considered to be bacteria that do not comprise flagellin or flagella that still possesses all the antigenic determinants. It is not necessary to remove all antigenic determinants: it suffices to delete one of the antigenic determinants. Screening for antibodies against this antigenic determinant in the serum of seropositive animals [then] would then allow discrimination between marker-vaccinated and wild type infected animals. Such antibodies would not be found in vaccinated animals, [whereas they]

but would be present in naturally infected animals. Screening can easily be done with simple diagnostic tools on a routinely basis as will be described below.

[0012] Much is currently known about the synthesis of flagellin and the subsequent maturation into flagella. ([E.g.]For example, in *Escherichia coli* and *Salmonella typhimurium*. Chapter 10: Flagella and motility. Eds. Frederick C. Neidhardt et al. 2nd. ed. ISBN 1-55581-084-5 (1996) and in Macnab, R.M.; Genetics and biogenesis of bacterial flagella (Annual Review of Genetics 26:131-158 (1992)). The process of flagellar biogenesis requires the concerted action of a large number of genes, not only the gene encoding the flagellin but also a large number of genes involved in the synthesis of the flagellum and the flagellar motor. In principle, there are two approaches for making bacteria according to the present invention. First[ of all], mutations can be made in the gene encoding the flagellin protein in order to mutate one or more antigenic determinants. Secondly, one or more genes involved in the biogenesis of the flagellum can be mutated. This will prevent the biosynthesis of flagellin or even the whole flagellum, and in many cases even the transport of the flagellin through the bacterial membrane. In the case that no flagella are produced, antigenic determinants determined by the specific folding of the flagellin in flagella will be absent. If the flagellin itself is not transported through the membrane and thus remains inside the bacterium, there will be no induction of antibodies against flagellin. All kinds of genes or gene clusters known in the art to be involved in assembly and export of such flagella, as the Morphological Assembly Pathway and a Flagellum-specific Export Pathway, can be targets for mutation[. They] as they all lead either to bacteria lacking flagellin or at least flagella. For the sake of clarity, all pathways involved in the whole process of synthesis of the final flagella are further referred to as flagellar biogenesis pathways.

[0013] Therefore, in a preferred embodiment, the bacterium is not capable [to induce]of inducing antibodies against at least one antigenic determinant of flagellin or flagella due to a mutation in a gene of the flagellar biogenesis pathway.

[0014] From a practical point of view, it may however be desirable to delete (part of) the whole flagellin gene from the bacteria to be used in the vaccine, simply by deletion of the gene encoding the flagellar protein. The genes encoding the flagellar proteins of the various *Salmonella* species are known. They are all very closely related, and therefore highly

homologous. Flagellin genes have *i.e.* been described for *Salmonella enterica* (Li, J. et al., Proc. Natl. Acad. Sci. 91, 2552-2556 (1994)), *Salmonella enteritidis* (Selander, R.K. et al., J. Bacteriol. 174, 3587-3592 (1992)), *Salmonella dublin* (Masten, B.J. and Joys, T.M., J. Bacteriol. 175, 5359-5365 (1993)), *Salmonella typhimurium* (de Vries, N. et al., Appl. Environ. Microbiol. 64, 5033-5038 (1998)), *Salmonella abortus-equi* (Hanafusa, T. et al., Mol. Gen. Genet. 236, 260-266 (1993)). Flagellin genes of novel *Salmonella* species can easily be found on the basis of their homology with all existing and known *Salmonella* flagellin genes: standard [hybridisation]hybridization techniques suffice for locating the flagellin gene.

[0015] There are two flagellin genes in wild-type flagellum-bearing *Salmonella* bacteria. Only one of these genes is switched on, *i.e.*, produces flagellin at any given time. Due to a mechanism called flagellar phase variation, at a time scale on the order of  $10^3$  to  $10^5$  generations the genes change roles so that the non-expressed one becomes expressed and vice versa. ([E.g.]For example, described in *Escherichia coli* and *Salmonella typhimurium*. Chapter 10: Flagella and motility. Eds. Frederick C. Neidhardt et al. 2nd. ed. ISBN 1-55581-084-5 (1996)). In order to avoid strains according to the invention [to start express]that start expressing flagellin after a certain number of generations, both flagellin genes must be made non-functional. Alternatively, if the phase switching mechanism is made non-functional it suffices to knock out the flagellin gene that is expressed at that time.

[0016] A non-functional gene is a gene that [does ]no longer [encode]encodes a flagellin. This can be a gene from which a part of the coding sequence has been removed that encodes an antigenic determinant.

[0017] One possible way of making the flagellin gene or any of the other known genes involved in flagellum-biosynthesis non-functional is by means of classical methods such as the treatment of wild-type bacteria flagella-producing bacteria with mutagenic agents such as base [analogues]analogs, treatment with ultraviolet light, or temperature treatment (Anderson, P. 1995. Mutagenesis, p 31-58 in Methods in Cell Biology 48. H.F. Epstein and D.C. Shakes (Eds)). Other methods for making flagella-less mutants of various bacteria of which the wild type has flagella, have been described *i.e.* by Liu, S. L. et al. (Infect. Immun. 1988 Aug; 56(8): 1967-

73), Haas, R. et al., (Mol. Microbiol. 1993 May; 8(4): 753-60) and Graf, J. et al. (J. Bacteriol. 1994 Nov; 176(22): 6986-91).

[0018] Selection for flagella-less bacteria is very easily done by light microscopic analysis for the absence or presence of flagella. Selection for bacteria lacking at least one antigenic determinant of flagellin or flagella can be done by means of binding assays with monoclonal antibodies against flagellin or flagella. Such anti-flagellar or anti-flagellin monoclonal antibodies can be made according to standard techniques, *i.e.* by [immunising]immunizing (with flagella) inbred mice by techniques known in the art (Kohler and Milstein, *Nature*, 256, 495-497, 1975). Monoclonals thus obtained can be used in binding assays with the mutated bacteria, and those bacteria not binding to a specific monoclonal are the bacteria lacking at least one antigenic determinant of flagellin or flagella.

[0019] The nature of the mutation caused by classical mutation techniques is unknown. This may be a point mutation which may, although this is unlikely to happen, eventually revert to wild-type. Therefore transposon mutagenesis is a good alternative. Mutation by transposon mutagenesis, is also a mutagenesis-technique well-known in the art. This is a mutation accomplished at a [localised]localized site in the chromosome.

[0020] A possibility to introduce a mutation at a predetermined site, [rather] deliberately rather than randomly, is offered by recombinant DNA-technology. Such a mutation may be an insertion, a deletion, a replacement of one nucleotide by another one or a combination thereof, with the only proviso that the mutated gene no longer encodes functional flagellin. Such a mutation can[ e.g.], for example, be made by deletion of a number of base pairs. Even very small deletions such as stretches of 10 base pairs can already render flagellin non-functional. Even the deletion of one single base pair may [already] lead to a non-functional flagellin, since as a result of such a mutation, the other base pairs are no longer in the correct reading frame. Each deletion or insertion of a number of base pairs indivisible by three causes such a frame shift. More preferably, a longer stretch is removed[ e.g.], for example, 100 base pairs. Even more preferably the whole flagellin gene is deleted. It can easily be seen, that especially mutations introducing a stop-codon in the open reading frame, or mutations causing a frame-shift in the open reading frame are very suitable to obtain a strain which no longer encodes flagellin. Site-directed

mutagenesis is the method of choice for making a flagellin gene that lacks one or more specific antigenic determinants. Based upon the map of antigenic determinants made by Joys (Joys, T.M., SAAS Bulletin: Biochem. & Biotech. 4: 56-59 (1991)), mutant flagellin genes can be made from which one or more specific antigenic determinants have been removed. All recombinant DNA techniques for the construction of flagellin-negative mutants are well-known standard techniques. They relate to cloning of the flagellin-gene, modification of the gene sequence by site-directed mutagenesis, restriction enzyme digestion followed by re-ligation or PCR-approaches and to subsequent replacement of the wild type flagellin gene with the mutant gene (allelic exchange or allelic replacement). Standard recombinant DNA techniques such as cloning the flagellin gene in a plasmid, digestion of the gene with a restriction enzyme, followed by endonuclease treatment, re-ligation and homologous recombination in the host strain, are all known in the art and described *i.e.* in Maniatis/Sambrook (Sambrook, J. *et al.* Molecular cloning: a laboratory manual. ISBN 0-87969-309-6). Site-directed mutations can[ e.g.], for example, be made by means of in vitro site directed mutagenesis using the Transformer® kit sold by Clontech. PCR-techniques are extensively described in (C. Dieffenbach & G. Dveksler[Dreksler]; PCR primers, a laboratory manual. CSHL Press, 1995, ISBN 0-87969-447-3[5] [(1995)]).

[0021] Therefore, in a [more ]preferred form, [this]an embodiment of the invention relates to a bacterium that is not capable [to express]of expressing a flagellin due to a mutation in the flagellin gene.

[0022] Most preferably, the bacteria are selected from the group consisting of *S. typhimurium*, *enteritidis*, *choleraesuis*, *dublin*, *typhi*, *abortus-ovi*, *abortus-equi*, *paratyphi A* and *B*, *derby*, *hadar*, *heidelberg*, *agona* and *arizonae*.

[0023] It is another object of the present invention to provide for the first time suitable marker vaccines for the protection of humans and animals against Salmonellosis. Vaccines according to the invention have as a characteristic feature that they comprise bacteria as defined above or antigenic material thereof, and a pharmaceutically acceptable carrier. The main advantage of vaccines according to the present invention is, that humans and animals vaccinated therewith can be discriminated from both non-vaccinated humans and animals and wild type *Salmonella* infected humans and animals on the basis of their antibody panel.

**[0024]** Vaccines according to the invention can be in a live attenuated or an inactivated form. In both cases the absence of at least one antigenic determinant of flagella or flagellin will result in an antibody panel after vaccination that can easily be discriminated from that induced after wild-type infection.

**[0025]** Inactivated vaccines have the advantage over [life]live vaccines that they are inherently safe. Therefore, one preferred form of the invention relates to vaccines in which the bacteria are in an inactivated form.

**[0026]** Vaccines according to the invention that are based upon live *Salmonella* bacteria [however] have an advantage over vaccines comprising inactivated bacteria [in that] because they better mimic the natural infection and therefore trigger the immune system in a better way. They also have an additional important advantage as explained hereafter. The development of live attenuated vaccines in general is difficult and time consuming. Moreover, fine-tuning the degree of attenuation is complex: high virulence causes disease, and low virulence induces insufficient protection. Surprisingly, vaccines according to the invention do not show significant differences in virulence when compared to their flagella-bearing counterparts. In other words, removal of the flagellin gene does not significantly change the level of attenuation. This has the unexpected advantage that both future and approved existing live attenuated *Salmonella* strains suitable for use in vaccines can be used in the present invention as soon as [the]they are made non-capable to induce antibodies against at least one antigenic determinant of flagellin or flagella. Therefore, in another preferred form, the vaccines according to the invention comprise live attenuated bacteria.

**[0027]** Given the large amount of vaccines given [nowadays] to both pets and farm animals, it is clear that combined administration of several vaccines would be desirable, if only for reasons of decreased vaccination costs. It is therefore very attractive to use live attenuated bacteria as a recombinant carrier for heterologous genes, encoding antigens selected from other pathogenic micro-organisms or viruses. Administration of such a recombinant carrier has the advantage that immunity is induced against two or more diseases at the same time. Live attenuated bacteria for use in a vaccine according to the present invention provide very suitable carriers for heterologous genes. In principle such heterologous genes can be inserted in the bacterial genome at any non-essential site.

[0028] Therefore, another embodiment of the invention relates to bacteria according to the invention in which a heterologous gene is inserted. Such a heterologous gene can, as mentioned above, [e.g.]for example, be a gene encoding an antigen selected from other pathogenic micro-organisms or viruses. Such genes can[ e.g.], for example, be derived from pathogenic herpesviruses ([e.g.]For example, the genes encoding the structural proteins of herpesviruses), Retroviruses ([e.g.]for example, the gpl60 envelope protein), adenoviruses and the like.

[0029] Also a heterologous gene can be obtained from pathogenic bacteria. As an example, genes encoding bacterial toxins such as [Actinobacillus pleuropneumoniae] Actinobacillus pleuropneumoniae toxins, [Clostridium] Clostridium toxins, outer membrane proteins and the like are very suitable bacterial heterologous genes.

[0030] Another possibility is to insert a gene encoding a protein involved in triggering the immune system, such as an interleukin, Tumor Necrosis Factor or an interferon, or another gene involved in immune-regulation.

[0031] The use of the flagellin gene as an insertion site has the advantage that there is no need to find a new insertion site for the heterologous gene and at the same time the flagellin gene is inactivated and the newly introduced heterologous gene can be expressed (in concert with the homologous bacterial genes). The construction of such recombinant carriers can be done routinely, using standard molecular biology techniques such as allelic exchange.

[0032] Thus, in a preferred [form of this ]embodiment, the heterologous gene is inserted in the flagellin gene. The heterologous gene can be inserted somewhere in the flagellin gene or it can be inserted at the site of the flagellin gene while this gene has been partially or completely deleted.

[0033] A vaccine according to the present invention also contains, in addition to the bacterium described above or antigenic material thereof, a pharmaceutically acceptable carrier. Such a carrier may be as simple as water, but it may[ e.g.], for example, also comprise culture fluid in which the bacteria were cultured. Another suitable carrier is[ e.g.], for example, a solution of physiological salt concentration.

[0034] The useful dosage to be administered will vary depending on the age, weight and animal vaccinated, the mode and route of administration and the type of pathogen against which vaccination is sought. The vaccine may comprise any dose of bacteria, sufficient to evoke an immune response. Doses ranging between  $10^3$  and  $10^{10}$  bacteria are[ e.g.], for example, very suitable doses.

[0035] Optionally, one or more compounds having adjuvant activity may be added to the vaccine. Adjuvants are non-specific stimulators of the immune system. They enhance the immune response of the host to the vaccine. Examples of adjuvants known in the art are Freunds Complete and Incomplete adjuvant, vitamin E, non-ionic block polymers, muramyldipeptides, ISCOMs (immune stimulating complexes, cf. for instance European Patent EP 109942), Saponins, mineral oil, vegetable oil, and Carbopol. Adjuvants, specially suitable for mucosal application are[ e.g.], for example, the *E. coli* heat-labile toxin (LT) or *Cholera* toxin (CT). Other suitable adjuvants are, for example, aluminum hydroxide, aluminum phosphate or aluminum oxide, oil-emulsions ([e.g.]for example, of Bayol F <sup>(R)</sup> or Marcol 52 <sup>(R)</sup>), saponins or vitamin-E solubilisate.

[0036] Therefore, in a preferred form, the vaccines according to the present invention comprise an adjuvant.

[0037] Other examples of pharmaceutically acceptable carriers or diluents useful in the present invention include [stabilisers]stabilizers such as SPGA, carbohydrates ([e.g.]for example, sorbitol, mannitol, starch, sucrose, glucose, dextran), proteins such as albumin or casein, protein containing agents such as bovine serum or skimmed milk and buffers ([e.g.]for example, phosphate buffer). Especially when such [stabilisers]stabilizers are added to the vaccine, the vaccine is very suitable for freeze-drying or spray-drying.

[0038] Therefore, in a [more] preferred form, the vaccine is in a freeze-dried or spray-dried form.

[0039] For administration to animals or humans, the vaccine according to the present invention can be given inter alia intranasally, by spraying, intradermally, subcutaneously, orally, by aerosol or intramuscularly. For application to poultry, wing web and eye-drop administration are additionally suitable.

[0040] Another embodiment of the invention relates to the use of bacteria according to the invention for the manufacture of a vaccine for the protection of humans and animals against infection with a *Salmonella* bacterium or the pathogenic effects of infection.

[0041] The invention also relates to methods for the preparation of a vaccine according to the invention. Such methods comprise the admixing of bacteria according to the invention or antigenic material thereof and a pharmaceutically acceptable carrier.

[0042] A diagnostic test for the screening for the absence/presence of *Salmonella* anti-flagellin or anti-flagella antibodies in sera can be [e.g.] a simple ELISA-test in which flagella or purified flagellin or a short polypeptide comprising an antigenic fragment thereof is coated to the wall of the wells of an ELISA-plate. Incubation with serum from humans or animals to be tested, followed by[ e.g.] incubation with a labeled antibody against the relevant human or animal antibody can then reveal the presence or absence of antibodies against the flagellin.

[0043] Another example of a diagnostic test system is[ e.g.], for example, the incubation of a Western blot comprising flagellin with serum of humans or animals to be tested, followed by analysis of the blot.

[0044] The diagnostic tests for the detection of antibodies against *Salmonella* flagellin are preferably in the form of a kit, comprising flagellin in a purified form. The flagellin could [e.g.] be purified through standard protein separation techniques over a suitable column. Another possibility is separation on a PAAGE gel followed by Western-blotting. On the Western-blot, the flagellin will form a specific band, separated from other *Salmonella* protein bands, and thus is also considered to be purified. Also, a pure form of the protein can be obtained by expressing flagellin-encoding nucleic acid sequences.

[0045] In principle, the easiest way of making such a diagnostic test system is to use purified whole flagellin as explained above. It is however [very well ]possible to use only part of the flagellin[. This], with the proviso that the fragment used still comprises an antigenic determinant of the protein. All antigenic determinants of the flagellin will induce antibodies by definition. Therefore, the use of a flagellin fragment comprising even one single antigenic determinant of the flagellin will be capable of binding to anti-flagellin antibodies.

[0046] A test that is capable of discriminating between serum from non-infected, infected and vaccinated humans or animals comprises [e.g.] a well A, coated with flagellin and a well B, coated with another *Salmonella* immunogen, or possibly whole *Salmonella* cells. Serum that reacts with wells A and B indicates a field infection or vaccination with a classical vaccine, whereas serum that reacts only with well B indicates that the animal tested is vaccinated with the marker vaccine.

[0047] The invention may be further understood by reference to the non-limiting examples set forth below.

## EXAMPLES

### **EXAMPLE 1**

[0048] *Salmonella typhimurium* strain STMP, an attenuated strain that has been tested as a live vaccine in poultry and pigs and provides good levels of protection was used as starting material for chemical mutagenesis.

#### **Chemical mutagenesis**

[0049] *S. typhimurium* STMP was grown on blood agar medium and checked for a positive O-antigen group B agglutination and H-antigen type 2 agglutination. One colony was inoculated into LB medium and incubated for 20 h at 37°C with aeration. Ten  $\mu$ l overnight culture was diluted in 10 ml LB (3 cultures) and incubated at 37°C for 6 h with aeration until the culture reached an O.D. at 600 nm of 0.5. A sample was taken to determine the number of viable bacteria. To each of the three 10 ml cultures 100  $\mu$ l trioxalen (chemical mutagens form Sigma; 3 mg/ml in DMSO) was added and the suspension was poured into a 10 cm Ø petri dish. The suspension was irradiated with U.V. (Transilluminator UVP; wavelength 365 nm) for 5, 10, or 15 min at a distance of 20 cm. Ten  $\mu$ l was transferred to 10 ml 0.9% NaCl, 1/10 dilutions were made and 100  $\mu$ l was plated on blood agar plates in order to determine the survival rate of bacteria in mutagenized cultures. Cultures with survival rates of approximately 3% and 20% were grown in

100 ml LB until an OD at 600 nm of 0.5. Bacteria were collected by centrifugation, resuspended in 5 ml LB and stored in 30% glycerol at 70°C.

**Selection for a non-motile, aflagellated mutant.**

[0050] The [mutagenised]mutagenized bacteria (3% survival; between 5,000 and 10,000 independent mutants) were inoculated from the glycerol stock onto 3 blood agar plates each. Bacteria were collected in LB and the OD at 600 nm was adjusted to 2.17. Six 10-fold dilutions were made in LB and then 50  $\mu$ l H2-antiserum (Difco) was added to 450  $\mu$ l bacterial suspension. This selection step with H-antiserum was included in order to enrich the suspension of mutants for aflagellated bacteria. This suspension was incubated at 37°C for 6 h while shaking (250 rpm) and then centrifuged for 1 min at 1000 rpm in an Eppendorf mini centrifuge in order to remove the agglutination-complex. From the supernatant 1, 10, and 100  $\mu$ l was plated on blood agar plates and incubated 20 h at 37°C.

**Results:**

**Identification of a non-motile mutant of *S. typhimurium* STMP.**

[0051] Selection for non-flagellated bacteria by incubation with H2 antiserum resulted in growth of approximately 40 colonies on plates (10<sup>6</sup> dilution) inoculated with the serum-treated mutant suspension (3% survival), whereas no colonies had grown on plates (10<sup>6</sup> dilution) inoculated with the serum-treated STMP suspension. This result indicated that mutants were present that did not agglutinate with the H2-antiserum, possibly being aflagellated. The 40 colonies were tested for H2-agglutination and for motility using light microscopy. All mutants were negative for H2-agglutination, but only one mutant appeared non-motile as observed by light microscopy. This non-motile mutant was positive for group B O-antigen agglutination. The mutant was named STM2000.

**In vitro stability of STM2000, a non-motile mutant of *S. typhimurium* STMP.**

[0052] The phenotypic stability of STM2000 was tested by 12 in vitro passages on blood agar plates. In the same experiment, the parent strain, STMP, was also passed 12 times.

These cultures were observed by light microscopy and all bacteria in the mutant-culture were still non-motile. STMP bacteria were motile, although not all cells showed the same level of motility. Electron microscopic comparison of STMP and STM2000 confirmed that no flagella were present on mutant bacteria.

**In vitro stability of STM2001, a non-motile mutant of *S. typhimurium* STMP.**

[0053] In a different experiment *S. typhimurium* SL3261 (Deposit number SGSC 439, Salmonella Genetic Stock Centre, University of Calgary, Alberta, Canada) was chemically [mutagenised]mutagenized with NTG and non-motile mutants were selected as described before. One mutant, STM2001, showed no reaction with a flagellin-specific monoclonal antibody. After 2D protein gel electrophoresis it was shown that STM2001 lacked the flagellin spot of 51 kDa and pI 4.7, as compared to its parent strain.

[0054] The genetic stability of this strain was tested by growing for at least 50 generations. After this period, still no revertants were found: all bacteria were still non-motile.

[Strain]

[0055] *S. typhimurium* strain STM2001 has been deposited with the Centraalbureau voor Schimmelcultures (CBS), [Oosterstraat 1]Uppsalaan 8, 3584 CT, P.O. Box [273]85167, [3740 AG Baarn]3508 AD Utrecht, The Netherlands, under accession-number CBS 108955.

**EXAMPLE 2**

**Experimental design**

[0056] Vaccines were prepared from a flagellated and a non-flagellated *S. enteritidis* ([S]"S.e.") phage type 4 strain. The bacteria were cultured in Tryptose Phosphate Broth, inactivated by the addition of formalin to a final concentration of 0.5%, followed by harvest of the bacterial cells by centrifugation. The cells were resuspended in phosphate buffer saline and formulated into water in oil emulsion vaccines at  $5 \times 10^9$  bacteria/ml. Five chickens were injected intramuscularly with the S.e. fla<sup>+</sup> vaccine and 5 chickens received the S.e. fla<sup>-</sup> vaccine. The animals were vaccinated with 0.5 ml vaccine at 14 and 18 weeks of age. At 22 weeks of age, the

chickens were bled, and serum was tested in a double antibody sandwich blocking ELISA system specific for antibodies to the g,m flagellin of S.e. (Zijderveld, F.G. van *et al.* (1993) Vet. Quart. 15, 135-137)

### **Animals**

[0057] Commercial laying type chickens, approximately 14 weeks of age were obtained from a *Salmonella* free flock.

### **Results:**

[0058] All 5 chickens that had received the S.e. fla<sup>+</sup> vaccine seroconverted in the g,m specific ELISA (blocking percentage >60%) whereas the 5 chickens vaccinated with the fla<sup>-</sup> vaccine remained seronegative.

## **EXAMPLE 3**

### **Experiment 1**

#### **Experimental design**

[0059] To assess safety, broilers were inoculated orally (1 ml), subcutaneously (0.5 ml) and intramuscularly (0.5 ml) with flagella-positive *Salmonella typhimurium* strain STMP, flagella-negative *Salmonella typhimurium* strain STM2000 or wild-type *S. typhimurium* [*/Salmonella typhimurium*]. The animals were observed for one week after inoculation followed by post-mortem examination of the surviving chickens.

### **Animals**

[0060] Commercial broilers, three weeks of age were obtained from a *Salmonella* free flock.

## **Results:**

[0061] Following inoculation, 8 out of 10 animals that had received wild-type *Salmonella typhimurium* died (Table 1). At necropsy, the 2 surviving chickens inoculated with the wild-type strain had swollen livers with necrotic foci, swollen spleens and pericardial edema. One of the STMP inoculated chickens had a slightly swollen liver and one chicken inoculated with STM2000 had a slightly swollen spleen. No further abnormalities were noted in those two groups.

Table 1

Group	STMP	STM2000	Wild-type
Dose (CFU/ml)	$10^{8.0}$	$10^{7.7}$	$10^{8.0}$
Mortality	0/10 <sup>a</sup>	0/10 <sup>a</sup>	8/10 <sup>b</sup>

Groups with different superscripts in a row differ ( $p<0.5$ , Fisher exact test)

## **Experiment 2**

### **Experimental design**

[0062] To test both safety and efficacy, broilers were inoculated orally at 3 and 15 days of age with either STMP or STM2000 followed by challenge infection with a tetracycline resistant wild-type *Salmonella typhimurium* strain at 22 days of age.

[0063] Safety was assessed by clinical observation after vaccination and determination of weight gain. Also, cloacal swabs were taken at days 10 and 22 to determine the presence of the vaccine strains in the intestinal tract. Swabs were used to inoculate Brilliant Green Agars (BOA) directly and after enrichment in Rappaport Vassiliades Broth (RVB). The animals were observed for one week after challenge infection followed by postmortem examination of the surviving chickens. The livers, spleens, cloacal swabs and swabs of the caecum contents of the surviving vaccinated chickens were cultured for the challenge strain by direct inoculation on BGA containing tetracycline (BGAtet). In addition, the swabs were incubated in an enrichment medium (buffered peptone water containing tetracycline) followed by plating on BGAtet.

## Animals

[0064] Commercial broilers, three days of age were obtained from a *Salmonella* free flock.

## Results:

[0065] No clinical abnormalities were observed after the oral vaccinations at 3 and 15 days of age. Also, the average weight gains in the vaccinated groups were not different from the control group (Table 3). Both strains were present in cloacal swabs of the vaccinated animals taken 7 days after vaccination (Table 2). The fact that a larger proportion of the chickens in the STMP inoculated group was culture positive after direct plating indicates that this strain [colonises]colonizes the intestinal tract in higher numbers than the STM2000 strain.

Table 2

Reisolation	STMP positive swabs		STM2000 positive swabs	
	Direct	Enrichment	Direct	Enrichment
Day 10	15/15 <sup>a</sup>	Not done	7/15 <sup>b</sup>	14/15
Day 22	10/15 <sup>a</sup>	15/15 <sup>A</sup>	14/15 <sup>a</sup>	14/15 <sup>A</sup>

Groups with different superscripts in a row differ ( $p<0.5$ , Fisher exact test)

[0066] One chicken in the STMP group died after challenge infection (7%), compared to no mortality in the STM2000 group and 80% mortality in the unvaccinated controls (Table 3). The surviving chickens in the control group also gained considerable less weight than the vaccines. The weight gain of the STM2000 vaccinated group after challenge infection was significantly higher than the weight gain of the STMP vaccinated group.

Table 3

Group	STMP	SM2000	Control
Dose d3	$10^{8.8}$	$10^{8.5}$	-
Dose d15	$10^{8.5}$	$10^{8.5}$	-
Weight gain d3-d22	$636 \pm 98^a$	$594 \pm 72^a$	$624 \pm 82^a$
S. t. challenge dose d22	$10^{8.0}$	$10^{8.0}$	$10^{8.0}$
Weight gain d22-d29	$299 \pm 45^a$	$339 \pm 42^b$	$31 \pm 39^c$
Mortality	1/15 <sup>a</sup>	0/15 <sup>a</sup>	8/10 <sup>b</sup>

Groups with different superscripts in a row differ ( $p<0.5$ , Fisher exact test (mortality) or two-sample t test (weight gain)

[0067] As shown in Table 4, there was no difference in the reisolation rates of the challenge organism from the spleen and liver. [Colonisation]Colonization of the intestinal tract, as judged by reisolation from cloacal swabs and caecum contents, was significantly lower in the group vaccinated with STM2000.

Table 4

<i>Salmonella typhimurium</i> (tet <sup>1</sup> ) positive				
Group	STMP vaccinated		STM2000 vaccinated	
Reisolation	Direct	Enrichment	Direct	Enrichment
Spleen	2/14 <sup>a</sup>	Not done	1/14 <sup>a</sup>	Not done
Liver	1/14 <sup>a</sup>	Not done	0/15 <sup>a</sup>	Not done
Cloaca	7/14 <sup>a</sup>	14/14 <sup>A</sup>	3/15 <sup>a</sup>	7/15 <sup>B</sup>
Caecum	13/14 <sup>a</sup>	14/14 <sup>A</sup>	5/15 <sup>b</sup>	9/15 <sup>B</sup>

Groups with different superscripts in a row differ ( $p<0.5$ , Fisher exact test)

## **EXAMPLE 4**

### **Experimental design**

[0068] To test efficacy, pigs were inoculated orally with STMP ( $10^{9.0}$  CFU) or STM2000 ( $10^{9.3}$  CFU) followed by an oral challenge infection with a streptomycin resistant wild-type *Salmonella typhimurium* strain ( $10^{11.0}$  CFU) 2 weeks later.

[0069] Shedding of the challenge strain was measured by culturing [faecal]fecal samples on days 5 and 8 post challenge. Approximately 5 grams of [faeces]feces were placed in peptone water and incubated for 2 hours. Then, serial ten-fold dilutions were made in RVB medium containing streptomycin, incubated overnight, followed by plating on Tryptose Phosphate Broth containing streptomycin. The maximum dilution containing the challenge strain was used to calculate the reisolation score (reciprocal of the maximum dilution positive). Eight STMP vaccinated pigs and 8 unvaccinated controls were used in a first experiment. In a second experiment 9 pigs were vaccinated with STM2000 and 9 unvaccinated controls were used.

### **Animals**

[0070] Six weeks old SPF pigs were used in both experiments.

### **Results:**

[0071] As shown in [table]Table 5, both vaccine strains were able to reduce [faecal]fecal shedding of the challenge strain significantly.

Table 5

	Average reisolation score	
	Day 5	Day 8
STMP (n=8)	$10^{0.9a}$	$10^{1.0a}$
Control (n=8)	$10^{6.4b}$	$10^{3.8b}$
STM2000 (n=9)	$10^{1.0A}$	$10^{1.3A}$
Control (n=9)	$10^{5.0B}$	$10^{4.9B}$

Groups with different superscripts differ ( $p < 0.5$ , Mann-Whitney U test)

#### Legend to the figures

Figure 1: protein profile analysis of STM2000 and its parental strain *S. typhimurium* STMP. Lane 1, 11 and 12 molecular weight markers as indicated; lane 2, 5 and 8, *S. typhimurium* STMP 12x passed on blood agar medium; lanes 3, 6, and 9, STM2000 12x passed on blood agar medium; lanes 4, 7, and 10, STM2000 2x passed on blood agar medium. Lanes 2, 3, and 4, total protein profile; lanes 5, 6, and 7, pellet after sonication and centrifugation; lanes 8, 9 and 10, supernatant after sonication and centrifugation. Proteins were stained with Coomassie Brilliant Blue.

Figure 2 Part (A): [photographs]photograph of electron microscopical examination of *S. typhimurium* STMP; Part ([A]B)[ and]: photograph of electron microscopical examination of its non-motile mutant STM2000[ (B)].

## Claims

What is claimed is:

## ABSTRACT OF THE DISCLOSURE

The present invention relates to *Salmonella* bacteria for use as a vaccine. The invention also relates to vaccines based thereon that are useful for the prevention of microbial pathogenesis. Further, the invention relates to the use of such bacteria or the manufacture of such vaccines. [Finally, the]The invention also relates to methods for the preparation of such vaccines.

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